Interference with the removal of oxaloacetate through citrate formation would naturally impair the catalytic oxidation of pyruvate. The inhibition of oxygen uptake stimulated by aspartate may well be another consequence of the same effect. A similar explanation can be offered for the inhibitory effect on glutamate oxidation, since oxobutyrate inhibits the removal of α-oxoglutarate as well.

α-Oxobutyrate, which inhibits the utilization of α-oxoglutarate, presumably enhances its formation from added citrate. Montgomery & Webb (1956) have reported a similar effect with another inhibitor of oxoglutarate oxidation, namely parapyruvate, in the presence of which the unaffected sections of the Krebs cycle proceed at a more rapid pace.

SUMMARY

1. α-Oxobutyric acid depresses the endogenous oxygen consumption of rat-liver homogenates.

2. Respiration stimulated by citrate, α-oxoglutarate, succinate, fumarate, malate, oxaloacetate, glutamate, aspartate or pyruvate is also depressed, the extent of this varying with the substrate used. It is maximal with oxaloacetate and minimal with citrate.

3. Inhibition has been observed at two sites in the cycle: (i) between oxaloacetate and citrate and (ii) between α-oxoglutarate and succinate. α-Oxobutyrate decreases citrate formation from added oxaloacetate and also removal of added α-oxoglutarate.

4. The implications of these findings are discussed.

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The Determination of Human-Serum-Cholinesterase Activity with *o*-Nitrophenyl Butyrate

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The increasing use of organophosphorus compounds in agriculture, forestry and medicine has placed great emphasis on the need for a rapid and reasonably accurate method for determining human-serum-cholinesterase activity. The present paper describes such a method based on the use of *o*-nitrophenyl butyrate as substrate. Cholinesterase liberates *o*-nitrophenol under easily reproduced, relatively non-critical conditions. The *o*-nitrophenol can be measured spectrophotometrically, and only a small volume (0·004 ml.) of serum is needed for each determination.

The effect of the orientation of the nitro group of nitrophenyl carboxylic esters on the cholinesterase activity was studied. Previously it had been shown that the A-type esterases in sheep serum hydro-

lysed *ortho*-orientated esters at a slower rate than the *meta* and *para* isomers (Main, 1960). The A-esterases in human serum showed a similar specificity. In the present work it was observed that cholinesterase hydrolysed *ortho*-nitro esters more rapidly than the corresponding *para* esters. Aldridge (1953) has shown that human serum contains only A-type esterases and cholinesterases. In addition he has shown that A-type esterases hydrolyse acetate esters more readily than butyrate esters, whereas Adams & Whittaker (1949) have shown that human-serum cholinesterase hydrolyses butyrates in preference to acetates. The application of these specificity studies has resulted in the synthesis of a compound, *o*-nitrophenyl butyrate, which is highly specific for cholinesterase in human serum.

EXPERIMENTAL

Materials

Preparation of nitrophenyl esters. *p*- and *m*-Nitrophenyl esters were prepared according to the Aldridge (1953) modification of the method of Huggins & Lapides (1947). *o*-Nitrophenyl acetate was a commercial preparation.

Preparation of o-nitrophenyl butyrate. This could not be prepared by the method of Huggins & Lapides (1947). Accordingly, the procedure given below was developed.

The sodium salt of *o*-nitrophenol was prepared by adding twice the stoicheiometric amount of a 75% (w/v) NaOH solution to a 25% (w/v) solution of *o*-nitrophenol in methanol at room temperature. The sodium salt was precipitated with about 90% yield. It was filtered on a Büchner funnel and washed three times with a small volume of methanol, once with ether, and dried in the air.

The sodium salt (16·2 g., 0·1 mole) was mixed with 30 ml. of wet benzene in a round-bottom flask and shaken to form a slurry. Butyryl chloride (14·5 ml., 0·14 mole) was added slowly with shaking at room temperature. As the reaction proceeded, the colour changed from red to yellow and NaCl was precipitated. When all the butyryl chloride had been added, the flask was placed under a reflux condenser, and the mixture was refluxed gently for 1 hr. After cooling, 3 vol. (approx. 150 ml.) of ether were added, and the solution was transferred to a 500 ml. separating funnel.

The organic phase was washed twice with water, three times with 1% aq. $NaHCO_3$, once with 1% citric acid and twice with water. The volume of each wash was about 200 ml. The organic phase was then transferred to a 250 ml. conical flask, and after drying over Na_2SO_4 (about 20 g.), it was filtered into a distillation flask, and the benzene ether components were distilled off under a partial vacuum, (approx. 100 mm. Hg). The yield from the sodium salt was about 85%.

The *o*-nitrophenyl butyrate was contaminated with *o*-nitrophenol. This was removed by recrystallizing the *o*-nitrophenyl butyrate three or four times from a 25% (v/v) solution in diethyl ether at the temperature of a solid CO_2–acetone bath. The freezing point of *o*-nitrophenyl butyrate was too low to permit crystallization at higher temperatures. Crystallization had to be initiated by seeding with crystals of *o*-nitrophenyl butyrate. These were obtained from a previously frozen solution in methanol. Although crystallization from methanol was much more rapid than from ether, the crystals obtained were heavily contaminated.

The freezing point of the purified *o*-nitrophenyl butyrate appeared to be about −20°; but unless the liquid was seeded, it remained liquid at this temperature. The ether was decanted from the crystal crops, and traces were removed from the last crop by evaporation under vacuum (60 mm. Hg) at 50°. The *o*-nitrophenyl butyrate decomposed before it would distil at 760 mm. Hg, and even under low pressure (2–3 mm. Hg) it tended to decompose and give *o*-nitrophenol in the distillate. The *o*-nitrophenyl butyrate prepared as described was 99·5–99·7% pure and contained 0·4–0·2% of *o*-nitrophenol. This amount of *o*-nitrophenol gave a negligible reagent blank at the *o*-nitrophenyl butyrate substrate concentrations normally used.

The density of *o*-nitrophenyl butyrate at 25° was 1·147.

Phosphate buffer. Most reactions were carried out at pH 7·6 in 50 mM-phosphate buffer. For convenience a 500 mM-solution was prepared by adding 9 g. of NaH_2PO_4,H_2O and 160 g. of $Na_2HPO_4,12H_2O$ and diluting to 1 l. This solution was diluted tenfold when required.

Diisopropyl phosphorofluoridate. Diisopropyl phosphorofluoridate (DFP) (0·10 ml.) was diluted to 25 ml. with propan-2-ol. The concn. of DFP was about 25 mM.

Buffered substrates. According to Aldridge (1953), the solubility of *p*-nitrophenyl carboxylic esters is about one-tenth of the values given by Huggins & Lapides (1947). The solubility of *p*-nitrophenyl acetate would then be approx. 3 mM, and of the *p*-nitrophenyl butyrate, 1 mM at 25°. Solution of these esters required special attention. It has been observed that *o*- and *p*-nitrophenyl acetates in concentrations as high as 5 mM could be prepared if a suitable procedure such as the one below was followed.

Stock solutions of the esters (usually 0·5 M) in methanol or other convenient alcohol were prepared. These solutions were stable for weeks if the alcohol used was first adjusted to about pH 5 with acetic acid.

Appropriate volumes of stock solution were blown into the aqueous medium with the tip of the pipette below the liquid surface as recommended by Huggins & Lapides (1947). Solution was more effective if the stock was blown into salt-free water. When concentrations of about 5 mM were desired, preheating the water to 32° was necessary. The dissolved substrate solution was then cooled and a suitable volume of concentrated phosphate buffer and acacia was added.

Human serum. Samples of human blood were withdrawn from volunteers by hypodermic syringe and defibrinated by stirring with a glass rod to which the clot adhered. The blood was collected in polyethylene test tubes and immediately centrifuged to separate the serum. The serum was stored frozen.

For routine testing, samples of blood were taken by pricking the finger and collecting in a capillary tube, which had previously been soaked in 0·1% heparin solution and dried. The plasma was separated by sealing one end of the capillary and centrifuging. Approx. 0·05 ml. of plasma was obtained in this way.

Determination of enzyme activity

Spectrophotometric method. The rate of *o*-nitrophenyl butyrate hydrolysis was determined by measuring the concentration of *o*-nitrophenol liberated in a given time either with a Hilger Uvispek or with a Beckman DK-2 ratio

recording spectrophotometer, equipped with time drive and temperature-controlled cell compartment.

In the presence of *o*-nitrophenyl butyrate, *o*-nitrophenol can be measured at two absorption peaks as shown in Fig. 1. One peak at 348 mμ is the absorption of the un-ionized form, and the other at 414 mμ is the absorption of the ionized oxide form. Since the p*K* of *o*-nitrophenol is pH 7·1, the ionized form predominates above and the un-ionized below this pH. The isosbestic point, at which absorption is independent of pH, was 371 mμ. When the Hilger Uvispek was used, the *o*-nitrophenol concentration was a linear function of absorption up to an extinction of 1·5.

The absorption spectra of *o*-nitrophenyl butyrate was independent of the pH. As shown in Fig. 1, it absorbed at and below the wavelength of the *o*-nitrophenol isosbestic point.

In the present work, it proved more convenient to measure *o*-nitrophenol concentrations at 414 mμ and pH 7·6 where the sensitivity is 1·5 as high as at the isosbestic point and where the *o*-nitrophenyl butyrate absorption was negligible. The sensitivity could be further increased by bringing the pH above 9, but the spontaneous hydrolysis of *o*-nitrophenyl butyrate at such pH values creates large reagent blanks. The *o*-nitrophenol absorption was calibrated for each new preparation of buffer, in order to correct for small pH variations. Colorimetric determinations were all carried out at 25° in a constant-temperature bath. Careful temperature control, within ±0·1°, was necessary to achieve the highest accuracy.

The cholinesterase-catalysed hydrolysis rates of *p*-nitrophenyl acetate and *p*-nitrophenyl butyrate were also determined. The liberated *p*-nitrophenol was measured at its isosbestic point (345 mμ). The absorption spectra of *p*-nitrophenol and of its acetate are given by Bergmann, Rimon & Segal (1958) who reported that the *p*-nitrophenol isosbestic point was 348 mμ.

A-Esterase activities were measured with serum dilutions which had previously been incubated with 0·1 mM-DFP. The reagent blank contained an equivalent amount of serum dilution which had previously been acidified and boiled to destroy all enzymic activity. The A-esterase activity was the difference in absorption between the reaction medium containing the DFP serum dilution and the boiled enzyme blank.

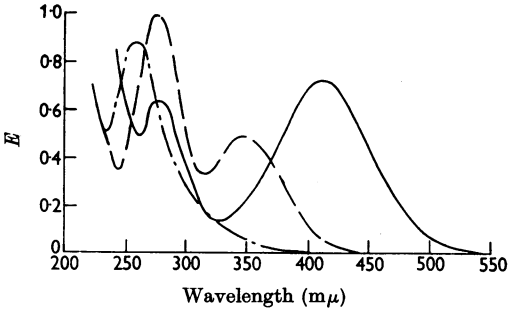

Fig. 1. Absorption spectra of *o*-nitrophenol and of *o*-nitrophenyl butyrate. The *o*-nitrophenol and *o*-nitrophenyl butyrate concentrations were 0·15 mM. The *o*-nitrophenol (un-ionized) spectrum at pH 4·5 (– –) and the *o*-nitrophenyl butyrate spectrum (– · –) were determined in 20 mM-acetate buffer. The *o*-nitrophenol (ionized) spectrum at pH 9 (—) was determined in 20 mM-borate buffer. The spectra were determined at 25° with the Beckman DK-2 ratio recording spectrophotometer.

Cholinesterase activity was measured against a reagent blank containing an equivalent amount of serum, which had previously been incubated with 0·1 mM-DFP to inhibit all the cholinesterase activity. The difference between the absorption of the solution containing uninhibited serum and inhibited serum, after a suitable digestion time, was a measure of the cholinesterase activity. When the Hilger Uvispek was employed, the cholinesterase activity was terminated by adding DFP to the uninhibited reaction medium. Typically, 0·05 ml. of 25 mM-DFP was added to 5 ml. of buffered substrate and an appropriate volume of serum contained in a 10 ml. volumetric flask. Both inhibited and uninhibited reaction media were then diluted to 10 ml. with 50 % (w/v) ethanol and read at 414 mμ at any convenient time. Mg^{2+} ions (10 mM) did not affect the hydrolysis of *o*-nitrophenyl butyrate by serum cholinesterase. The *o*-nitrophenol absorption was calibrated in the presence of alcohols at the final concentration used in the reaction medium.

Manometric method. A modification of the method of Aldridge (1953) was used for the determination of *o*-nitrophenyl butyrate activity. Generally 0·5 ml. of an enzyme dilution was placed in the side arm and 2·5 ml. of buffered substrate was placed in the centre well. The final concentration of the reagents, exclusive of substrate, was 26 mM-$NaHCO_3$, 100 mM-NaCl and 0·2 % of acacia. The flasks were gassed with CO_2+N_2 (5:95) at room temperature for 15 min. before being placed in the 37° bath. Readings were taken 2 min. after tipping and at 5 min. intervals after the first 5 min. reading. The activity was calculated from the initial velocity, and usually the readings for the first 15 min. approximated to a straight line.

RESULTS

Specificity. The substrate specificity patterns of human-serum and horse-serum cholinesterase (as judged by eserine inhibition) are compared with that of a purified cholinesterase preparation from horse serum (Strelitz, 1944, stage 2), in Fig. 2, which clearly shows the similarity of the patterns. *o*-Nitrophenyl butyrate was hydrolysed 2·4 times as rapidly as acetylcholine by human cholinesterase, compared with 1·8 times by horse-serum and purified horse-serum cholinesterases.

Within experimental limits, eserine (10 μM), and paraoxon (10 μM) inhibited the same percentage (about 97 %) of the human-serum activity hydrolysing *o*-nitrophenyl butyrate. The remaining activity was therefore attributed to A-esterases. This result was consistent with the observation of Aldridge (1953) that human serum does not contain B-esterases.

Inhibition by eserine was also determined by spectrophotometric analysis. 0·1 mM-DFP inhibited 96·8 ± 1·3 % of the enzymic hydrolysis of *o*-nitrophenyl butyrate compared with 97·4 ± 1·7 %

inhibition by 0·03 mM-eserine. The eserine pI_{50} with *o*-nitrophenyl butyrate was 8·1.

These results, and the similarity of the substrate specificity patterns of human-serum and horse-serum cholinesterases, suggested that in human serum cholinesterase was responsible for most of the enzymic hydrolysis of *o*-nitrophenyl butyrate.

The cholinesterase and A-esterase activities of different human sera towards *o*-nitrophenyl butyrate were compared, and the results are given in Table 1. Although the activities varied widely between different sera, in the serum with the lowest cholinesterase to A-esterase ratio cholinesterase was responsible for 96·3 % of the activity.

The cholinesterase and A-esterase specificity towards a number of nitrophenyl esters is compared in Table 2. These results suggested that A-esterase was most active towards *para*-orientated nitro esters substituted with acetate, whereas cholinesterase was more specific towards *ortho* esters and butyrates. The possibility of selecting a nitrophenyl ester with an orientation and substitution reasonably specific for any esterase to be determined was also suggested by these results. For example, whereas cholinesterase was responsible for 97 % of the *o*-nitrophenyl butyrate hydrolysis, A-esterases accounted for about 80 % of the *p*-nitrophenyl acetate hydrolysis under similar conditions.

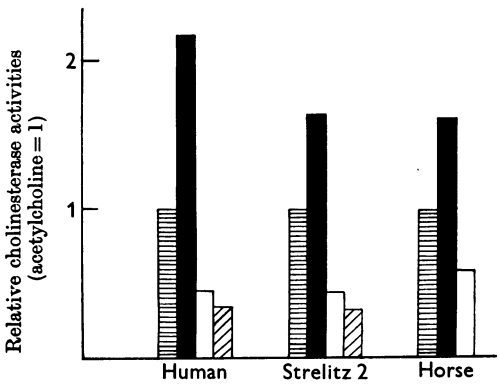

Fig. 2. Comparison of three cholinesterase substrate specificity patterns; human serum, purified preparation (Strelitz, 1944, stage 2) from horse serum, and horse serum. Activities were determined manometrically at 37° and pH 7·4. The substrate concentrations were: acetylcholine, 30 mM (▤); *o*-nitrophenyl butyrate, 4·2 mM (■); tributyrin, 16 mM (▨); *p*-nitrophenyl acetate, 4·2 mM (□). Final concentrations in digest medium, exclusive of inhibitors and substrate were: NaCl, 0·1 M; $NaHCO_3$, 26 mM; acacia, 0·2 %. The serum-cholinesterase activity was determined by the difference in the activity of uninhibited serum and serum inhibited by addition 30 min. previously of 60 μM-eserine. Final eserine concentration was 10 μM. The activity towards acetylcholine was for human sera, 66 ± 4, and horse sera, 81 ± 1 μl. of CO_2/ml. of serum/min.

Table 3 gives a comparison of the K_m and $V_{max.}$ values of *o*-nitrophenyl butyrate, *o*-nitrophenyl acetate, *p*-nitrophenyl butyrate and *p*-nitrophenyl acetate obtained with human-serum cholinesterase. The specificity suggested by the relative activities towards these compounds was further confirmed by the K_m values. These results suggested that cholinesterase activity was affected more by the orientation of the nitro group than by the nature of the acyl substituent. Thus, substituting acetate for butyrate in the *ortho* compounds decreased the activity by half and raised the K_m by a factor of 2·5. Substituting *p*-nitrophenyl for *o*-nitrophenyl decreased the activity by a third and raised the K_m by a factor of almost 8.

For routine activity determinations, it is desirable to determine the activity at substrate concentrations where the activity is independent of the concentration. With some of the nitrophenyl esters this was not possible because of low solubilities and high K_m values. However, the *o*-nitrophenyl butyrate K_m of cholinesterase was sufficiently low and the *o*-nitrophenyl butyrate solubility sufficiently high for this condition to be easily realized.

Stability of nitrophenyl esters. The spontaneous hydrolysis rates of a number of nitrophenyl carboxylic esters are compared in Table 4. Spontaneous hydrolysis appears to be a second-order reaction, depending on the concentration of both the ester and the hydroxyl ion. When the hydroxyl ion concentration is constant, as in a buffered medium, the hydrolysis rate may be treated as a first-order reaction and comparison of rates made on this basis.

The *ortho*-nitrophenyl esters were from 1·5 to 3·8

Table 1. *Variation between individual sera of the cholinesterase and A-esterase activities towards o-nitrophenyl butyrate*

Each determination was made in duplicate on the DK-2 recording spectrophotometer. For cholinesterase determinations 0·010 ml. of serum was used and for A-esterase determinations 0·050 ml. of serum. Concn. of *o*-nitrophenyl butyrate was 1 mM, pH 7·6, temp. 25°.

Activity (μmoles of *o*-nitrophenol/ml. of serum/min.)		Cholinesterase
Cholinesterase	A-Esterase	A-Esterase
2·84	0·049	58
2·69	0·087	31
2·48	0·080	31
2·97	0·053	56
2·14	0·070	31
1·98	0·033	60
2·80	0·105	27
2·80	—	—
Average ± S.D.		
2·59 ± 0·36	0·068 ± 0·025	42 ± 15

Table 2. *Comparison of the activities of human-serum cholinesterase and A-esterases toward nitrophenyl esters*

The activity was determined with 1 mM-substrates at pH 7·6 in 0·05 M-phosphate buffer at 25°, with the DK-2 spectrophotometer according to the method in Experimental section. The *p*-nitrophenol liberated was followed at 400 mμ. For cholinesterase activities 0·010 ml. of serum was used; for A-esterase activities 0·050 ml. of DFP-treated serum was used. An equivalent amount of pre-boiled serum was added to the A-esterase determination reagent blank.

Substrate	Activity (μmoles of nitrophenol liberated/ml. of serum/min.)		Cholinesterase / A-Esterase
	Cholinesterase	A-Esterase	
o-Nitrophenyl butyrate	2·59*	0·068*	42
o-Nitrophenyl acetate	1·50†	0·78†	1·9
p-Nitrophenyl butyrate	0·66†	0·27†	2·41
p-Nitrophenyl acetate	0·32†	1·04†	0·31

* Average of results from Table 1.
† Pooled sera of four individuals.

Table 3. *Comparison of the human-serum cholinesterase $V_{max.}$ values and K_m values of four nitrophenyl esters*

The activities were determined with the Beckman DK-2 recording spectrophotometer at 25°, pH 7·6 in 0·05 M-phosphate buffer. Typically, five concentrations (1, 0·33, 0·2, 0·15 and 0·1 mM) of substrate were used to determine a K_m. The Dixon (1953) modification of the Lineweaver & Burk (1934) plot was used. Usually 0·010 ml. of serum was used in 5 ml. of buffered substrate. The reagent blank was identical with the activity solution, except that the serum used had been previously incubated with 0·1 mM-DFP.

Substrate	$V_{max.}$ (μmoles of nitrophenol/ml. of serum/min.)	K_m (mM)	Activity ratio (*o*-Nitrophenyl butyrate = 1)
o-Nitrophenyl butyrate	3·32	0·106	1
o-Nitrophenyl acetate	1·67	0·25	0·50
p-Nitrophenyl butyrate	1·16	0·80	0·35
p-Nitrophenyl acetate	0·60	1·08	0·18

times as stable as the corresponding *para* esters. The order of decreasing stability with respect to acyl substituent was butyrate > propioniate > acetate. For the purposes of routine activity determination, it was convenient to have a compound of reasonable stability at pH 7·6 in order to decrease possible reagent-blank errors to negligible proportions. From this point of view, *o*-nitrophenyl butyrate was the most suitable ester examined, with a half-life of 3 days under the conditions used.

In addition to spontaneous non-enzymic hydrolysis of these esters, there is a non-enzymic hydrolysis caused by the serum proteins which must also be considered when evaluating enzymic hydrolysis. As shown in Table 5, this hydrolysis was not proportional to the amount of serum used, nor was it linear with time. The rates given in Table 5 are initial rates, but the total hydrolysis in the time used for routine analysis (30 min.) was less than one-third of that suggested by the initial rate. When determinations were made with the DK-2 ratio recording spectrophotometer, the protein non-enzymic hydrolysis (together with the other non-enzymic and non-cholinesterase enzymic activities) were automatically cancelled by using a suitable reference blank. With the routine procedure, the non-enzymic serum-protein hydrolysis was decreased to negligible portions (Table 6).

Effect of butanol. Butan-1-ol activated the cholinesterase hydrolysis of *o*-nitrophenyl butyrate and inhibited the barely significant A-esterase activity. In the presence of 5% (v/v) butanol, all the measurable enzymic hydrolysis of *o*-nitrophenyl butyrate by human serum was attributable to cholinesterase. With mM-*o*-nitrophenyl butyrate, 5% (v/v) butanol increased the activity by a factor of 3·08 ± 0·21 as judged by duplicate determinations of seven different sera. The activity increased, from an average of 2·56 to 7·89 μmoles of nitrophenol liberated/ml. of serum/min., in the presence of 5% (v/v) butanol. The increase in activity was proportional to butanol concentration (Main, 1961). K_m increased from 0·11 mM in the absence of butanol to 0·46 mM in the presence of 5% (v/v) butanol. With the volume of serum used for routine determinations of *o*-nitrophenyl butyrate (0·004 ml.), the

Table 4. *Non-enzymic hydrolysis rates of various nitrophenyl carboxylic esters*

The initial concentration of each ester was 1 mM. Most solutions were prepared by diluting 0·20 ml. of a 0·50M-stock solution of ester in methanol to 100 ml. with 0·05M-phosphate buffer. With *p*-nitrophenyl acetate, 1·0 ml. of a 0·10M-methanol solution of *p*-nitrophenyl acetate was diluted to 100 ml. with buffer. The hydrolysis was followed with a DK-2 recording spectrophotometer over appropriate periods of time. The half-life $t_{\frac{1}{2}}$ was calculated assuming the hydrolysis was a pseudo first-order reaction. Temp., 25°; pH 7·6.

Ester	$t_{\frac{1}{2}}$ (min.)	$10^4 \times k^{-1}$ (min.)
o-Nitrophenyl butyrate	4500	1·5
o-Nitrophenyl propionate	1300	5·3
o-Nitrophenyl acetate	730	9·4
p-Nitrophenyl butyrate	1300	5·3
p-Nitrophenyl propionate	570	12
p-Nitrophenyl acetate	480	14

Table 5. *Non-enzymic hydrolysis of o-nitrophenyl butyrate and p-nitrophenyl acetate by previously acidified and boiled human serum*

A 1:10 dilution of human serum was acidified with 2N-acetic acid to pH 3·5 and was then placed in boiling water for 10 min. After cooling, the pH of the serum dilution was adjusted with 2N-NaOH to pH 7·3. The hydrolysis rates were determined with mM-substrate concentration at 25° and pH 7·6 on the DK-2 recording spectrophotometer. The control blanks were identical with the unknown samples except that no boiled serum was added.

Ester	Boiled serum used (ml.)	Hydrolysis, initial rate (μmoles of nitrophenol/ml. of serum/min.)
p-Nitrophenyl acetate	0·01	0·33
o-Nitrophenyl butyrate	0·01	0·26
o-Nitrophenyl butyrate	0·02	0·21
o-Nitrophenyl butyrate	0·05	0·17

Table 6. *Reproducibility of the method for determining human-serum cholinesterase with o-nitrophenyl butyrate*

The cholinesterase activities of a number of different human sera were determined at 25° according to the procedure in the text. Two different serum dilutions were made with each serum. The activity of each dilution was determined in duplicate, with a different preparation of buffered substrate. These determinations were made on the same day. The average activity obtained for this day is compared with that determined on a previous day to illustrate the day to day reproducibility. The *o*-nitrophenyl butyrate hydrolysis by factors other than cholinesterase was determined by first adding 0·05 ml. of 25 mM-DFP to 5 ml. of substrate solution and then adding the serum dilution. Otherwise the procedure was the same as that for determining total activity. S.D. of the duplicates with serum dilution A, ±2·5%; S.D. of the duplicates with serum dilution B, ±1·5%; S.D. between average of serum dilutions A and B, ±1·8%; S.D. between Day 1 and Day 2 results, ±1·6%; non-cholinesterase hydrolysis was 0·7%, equivalent to extinction of 0·0026±0·0027.

Extinction Serum dilution A		Extinction Serum dilution B			Activity (μmoles of nitrophenol/ml. of serum/min.)	
1	2	1	2	Non-cholinesterase hydrolysis	Day 1 (average of 4)	Day 2 (average of 2)
0·334	0·340	0·335	0·332	0·003	6·92	6·96
0·420	—	0·413	0·405	0·004	8·55	8·38
0·402	0·414	0·405	0·419	0·000	8·44	8·45
0·420	—	0·413	0·410	0·001	8·56	8·14
0·300	0·295	0·315	0·295	0·002	6·62	6·77
0·401	0·376	0·362	0·359	0·002	7·72	7·40
0·430	0·405	0·414	0·410	0·008	8·55	8·73

activity was proportional to time over the entire 30 min. digestion period when the final *o*-nitrophenyl butyrate concentration was 1·25 mM, but deviated slightly toward the end in the presence of a 0·83 mM-solution. The presence of the butanol appeared to increase the *o*-nitrophenyl butyrate solubility so that 1·5 mM concentrations were readily obtained. The activity was independent of the *o*-nitrophenyl butyrate concentration at 1·25 mM, so that variations in the concentration (1·3 ± 0·3 mM) did not result in measurable loss of accuracy.

Determinations were slightly more reproducible if the enzyme was diluted in buffer containing 5% (v/v) butanol and 0·1% of acacia before being added to the substrate.

Effect of pH. The effect of pH on the cholinesterase activity towards *o*-nitrophenyl butyrate was determined in the presence and absence of butanol with the results shown in Fig. 3. The activity was essentially independent of the pH between pH 7·2 and 8·8 in the presence of butanol. With no butanol the activity was independent of the pH above a pH of 7·4, but the plateau was extended beyond the range found in the presence of butanol. A pH of 7·6 was chosen for most activity determinations, because of the stability of the *o*-nitrophenyl butyrate (Table 4). Small fluctuations

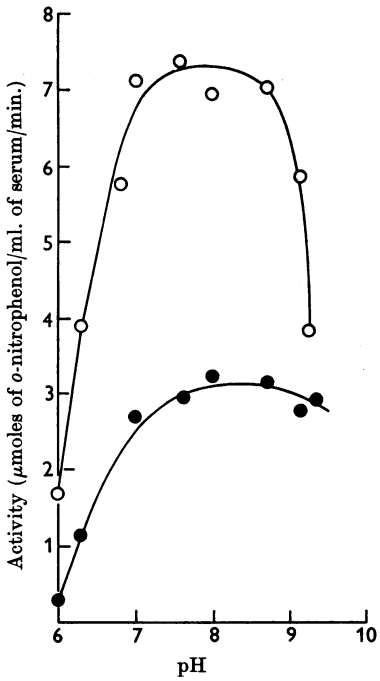

Fig. 3. Hydrolysis of *o*-nitrophenyl butyrate by human-serum cholinesterase as a function of pH in the presence of 4·2% of butanol (○) and without butanol (●). Activities were determined at 25°; 0·05M-phosphate buffers were used to cover the range from pH 6 to 7·8. From pH 8·0 to 9·25, 0·05M-borate buffers were used. The Hilger Uvispek was employed for all determinations. From 0·02 to 0·004 ml. of serum was used, depending on conditions. Digest time varied from 10 to 30 min. The *o*-nitrophenyl butyrate concentration was 1·0 mM.

in pH in this region would not appreciably affect the activity.

The cholinesterase activity toward *o*-nitrophenyl butyrate was proportional, within the limits of experimental error, to the amount of serum used over the range of volumes tested (0·001–0·100 ml.) at pH 7·6 and 25°.

Routine determination of human-plasma-cholinesterase activity

Buffered substrate. 0·5M-*o*-Nitrophenyl butyrate stock solution in methanol (0·3 ml.) was diluted to 100 ml. with 5% (v/v) butan-1-ol, pH 7·6, and 50 mM-phosphate buffer, according to the method described in the Experimental section. The concentration of *o*-nitrophenyl butyrate was 1·5 mM.

Plasma dilution. Blood was collected from finger punctures in capillaries as previously described. Plasma (0·02 ml.) was diluted to 5 ml. in ice-cold 5% butanol (v/v at 25°), pH 7·6 and 50 mM-phosphate buffer containing 0·1% acacia. Sahli blood pipettes calibrated 'to contain' were used to pipette the plasma.

The enzyme dilution was stable for at least 30 min. at room temperature. If delays greater than this were necessary before its use, preparation and storage in ice-cold buffers was advisable.

Procedure. Buffered substrate (5 ml.) was pipetted into two 10 ml. volumetric flasks (A and B). About 0·05 ml. (1 drop) of 25 mM-DFP was added to flask B and the flasks were placed in a constant-temperature bath at 25°. Plasma dilution (1·0 ml. containing 0·004 ml. of plasma) was added to flask A and B in that order to avoid DFP contamination of A. After 30 min., 0·05 ml. (1 drop) of 25 mM-DFP was added to flask A and both A and B were made up to volume with 50% (v/v) ethanol. The colour was read at 414 mμ with the contents of flask B as a reagent blank.

An example of the precision of the procedure is given in Table 6 where the duplicate determinations of a single plasma dilution are compared with the duplicate determinations of another dilution. In addition, the day-to-day precision is given. A measure of the non-enzymic plasma-protein hydrolysis is also given. This was obtained by measuring the difference in extinction between flask B and the contents of a flask identical with B, except that it had not been digested 30 min. before the addition of 50% (v/v) ethanol. The serum-protein non-enzymic hydrolysis was about 0·7% of the enzymic hydrolysis, but was so low as to be barely within the range of the spectrophotometer. This error was considerably less than the precision of the method (about ±2·5%).

Each determination required an estimated 2–3 min. manipulation. The activities were calculated in terms of μmoles of *o*-nitrophenol liberated/ml. of plasma/min. at pH 7·6 and 25°.

DISCUSSION

Huggins & Lapides (1947) described the use of *p*-nitrophenyl carboxylic esters as substrates in sensitive methods of determining activities of serum esterases. The nature and substrate specificity patterns of these esterases in sera and tissues has since been further clarified by a number of workers (for example Aldridge, 1953; Mounter & Whittaker, 1953; Bergmann, Segal & Rimon, 1957; Main, 1960).

Aldridge (1953) divided serum esterases into A and B classifications, neither of which included cholinesterases. A-Esterases hydrolysed acetate in preference to butyrate esters and were not inhibited by organophosphorus compounds. B-Esterases had the reverse specificity and, like cholinesterases, were inhibited by organophosphorus compounds. Cholinesterases and B-esterases were differentiated

by the inhibitor eserine. Main (1960) demonstrated that the orientation of the nitro group on the nitrophenyl part of these esters also influenced the activity. The A-esterases of sheep serum, for example, hydrolysed *m*-nitrophenyl acetate more rapidly than *p*-nitrophenyl acetate. The activity towards *o*-nitrophenyl acetate was only one-twenty-seventh of that towards the *meta*-isomer.

In the present work it has been shown that the substrate specificity of human-serum A-esterases is similar to that of sheep serum A-esterases. In contrast, human-serum cholinesterases hydrolysed *ortho*-isomers more rapidly than *para*-isomers. The results suggested that the nitro orientation, more than the nature of the acyl substituent, influenced the cholinesterase activity, but that the reverse was true for the A-esterases (Table 2).

In human serum, B-esterases are absent (Aldridge, 1953). Consequently, only the A-esterases and cholinesterase were concerned in the hydrolysis of esters. The present method was developed by applying and further extending this knowledge about the acyl and the isomeric nitro specificity of these chromogenic substrates.

For routine analysis, colorimetric methods generally have the advantage of sensitivity, rapidity and relative simplicity of manipulation over manometric, titrimetric and electrometric methods. In the past, colorimetric methods for the determination of serum-cholinesterase activity have not found wide acceptance for routine analysis. The phenyl benzoate method of Gomori (1949), for example, suffered from the disadvantage of low phenyl benzoate solubility and a relatively high K_m value. As a result, the activity was a critical function of the phenyl benzoate as well as of the enzyme concentration. In the author's experience, reproduction of a given substrate concentration was difficult to achieve, and consequently poor agreement was frequently observed between activities measured on different days. The β-carbonaphthoxycholine method of Ravin, Tsou & Seligman (1951) has the disadvantages of a low substrate solubility (0·12 mM) and a tedious diazotization and extraction step. In the present method activity was independent, within fairly wide limits, of the substrate concentration. In human serum or plasma, the substrate was highly specific and sensitive, particularly in the presence of butanol. Since *o*-nitrophenol could be measured directly, no colour-developing procedure was necessary. As a result, the method requires only 2 or 3 min. of manipulation per determination and is reasonably accurate (precision within $\pm 2{\cdot}5\%$).

SUMMARY

1. Human-serum and horse-serum cholinesterases hydrolysed *o*-nitrophenyl butyrate about twice as rapidly as acetylcholine. Human-serum cholinesterase had a greater affinity (K_m 0·11 mM), and greater activity towards *o*-nitrophenyl butyrate than toward any other nitrophenyl carboxylic ester tested.

2. Human-serum cholinesterase hydrolysed *ortho*-nitrophenyl carboxylic esters more rapidly than the *para* esters. A-Esterases had the reverse specificity.

3. In human serum, the cholinesterase was responsible for not less than 96% of the enzymic hydrolysis of *o*-nitrophenyl butyrate. A-Esterases accounted for the remaining activity.

4. In the presence of 5% (v/v) butan-1-ol, cholinesterase-catalysed hydrolysis of *o*-nitrophenyl butyrate was activated threefold, whereas the A-esterase activity was completely inhibited.

5. A rapid, sensitive, colorimetric method suitable for routine analysis was developed as a consequence of these investigations. Over conveniently wide ranges, the activity was independent of both the pH and substrate concentration. The *o*-nitrophenol liberated by hydrolysis was measured directly in the reaction medium. The colour was stable. The precision of the method was $\pm 2{\cdot}5\%$; each determination required 2–3 min. manipulation.

6. A method for the synthesis and purification of *o*-nitrophenyl butyrate is described.

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